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The *Drosophila* TRPA channel, Painless, regulates sexual receptivity in virgin females

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Abstract

Transient receptor potential (TRP) channels play crucial roles in sensory perception. Expression of the *Drosophila* *painless* (*pain*) gene, a homolog of the mammalian *TRPA1/ANKTM1* gene, in the peripheral nervous system is required for avoidance behavior of noxious heat or wasabi. Here we report a novel role of the Pain TRP channel expressed in the nervous system in the sexual receptivity in *Drosophila* virgin females. Compared with wild-type females, *pain* mutant females copulated with wild-type males significantly earlier. Wild-type males showed comparable courtship latency and courtship index toward wild-type and *pain* mutant females. Therefore, the early copulation observed in wild-type male and *pain* mutant female pairs is the result of enhanced sexual receptivity in *pain* mutant females. Involvement of *pain* in enhanced female sexual receptivity was confirmed by rescue experiments in which expression of a *pain* transgene in a *pain* mutant background restored the female sexual receptivity to the wild-type level. Targeted expression of *pain* RNAi in putative cholinergic or GABAergic neurons phenocopied the mutant phenotype of *pain* females. On the other hand, target expression of *pain* RNAi in dopaminergic neurons did not affect female sexual receptivity. In addition, conditional suppression of neurotransmission in putative GABAergic neurons resulted in a similar enhanced sexual receptivity. Our results suggest that Pain TRP channels expressed in cholinergic and/or GABAergic neurons are involved in female sexual receptivity.

Keywords

painless; TRP channel; mating; sexual receptivity; *Drosophila*; brain; GAL4/UAS; GAL80

Introduction

Genetic analysis can be used to identify genes required for specific behaviors and can lead to the identification of neural circuits and molecular mechanisms underlying the behavior. The fruit fly, *Drosophila melanogaster*, is an ideal model organism for investigating the molecular mechanisms or circuits underlying male and female sexual behaviors owing to the availability of vast genetic information and clearly defined behaviors. Genetic studies on *D. melanogaster* have resulted in the identification of genes regulating male courtship behaviors (Emmons & Lipton 2003; Hall 1994), and sites in the central nervous system (CNS) relevant to male courtship have been mapped (Broughton *et al.* 2004; Demir &

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Dickson 2005; Manoli *et al.* 2005; Sakai & Kitamoto 2006; Kimura *et al.* 2008). On the other hand, brain mechanisms that control sexual behavior in virgin females remain largely unknown.

In *Drosophila*, female sex pheromones evoke male courtship behavior (Greenspan & Ferveur 2000; Hall 1994; Jallon 1984; Tompkins 1984), and male courtship songs produced by wing vibrations stimulate the sexual receptivity in virgin females (Kyriacou & Hall 1982; Manning 1967; Tomaru *et al.* 2000; von Schilcher 1976). Receptive virgin females decide to accept courting males and copulate with them. In contrast, non-receptive females show a variety of rejection responses (Ewing 1983; Hall 1994; Spieth 1952). Several mutations (e.g., *paralytic*, *apterous*, *spinster*, and *icebox*) result in the low sexual receptivity in virgin females of *D. melanogaster* (Hall 1994; Kerr *et al.* 1997; Ringo *et al.* 1991; Suzuki *et al.* 1997; Tompkins *et al.* 1982), while the induction of a repressor isoform of *Drosophila* CREB, *dCREB2-b*, enhances the sexual receptivity in virgin females (Sakai & Kidokoro 2002). Thus, the sexual receptivity in virgin females can be enhanced or inhibited by various genetic manipulations.

Transient receptor potential (TRP) channels are evolutionarily conserved Ca²⁺-permeable cation channels that are expressed in a variety of cell types in invertebrates and vertebrates (Pedersen *et al.* 2005; Vriens *et al.* 2004). Cation influx through TRP channels depolarizes the neuronal membrane potential and increases the intracellular Ca²⁺ concentration (Ramsey *et al.* 2006). The *Drosophila* Painless (Pain) TRP channel is a homolog of the mammalian wasabi receptor, TRPA1/ANKTM1 (Corey 2003). The *pain* gene was first found to play an essential role in noxious thermal and mechanical perception in larval sensory neurons (Tracey *et al.* 2003), and Xu *et al.* (2006) have reported that *pain* is also required for thermal nociception in adult flies. A recent report indicates that the Pain TRP channel is activated by noxious heat *in vitro* (Sokabe *et al.* 2008). Furthermore, *pain*^{GAL4} containing a GAL4-enhancer trap insertion in the first exon of *pain* drives GFP reporter expression in adult taste receptor neurons, and *pain* is required for the behavioral response to wasabi in *Drosophila* (Al-Anzi *et al.* 2006). In this article, we show that the *pain* gene plays a critical role in the regulation of sexual receptivity in *Drosophila* virgin females, demonstrating for the first time its involvement in mating behavior.

Materials and Methods

Fly stocks

Drosophila melanogaster wild-type Canton-S (CS), *pain* mutants (*pain*¹, *pain*³ and *pain*^{GAL4}), UAS-*pain*, two UAS-*pain* RNAi lines (1A and 2A), several neural GAL4 lines [choline acetyltransferase (Cha)-GAL4-9A and Cha-GAL4-19B; tyrosine hydroxylase (TH)-GAL4; dopa decarboxylase (Ddc)-GAL4; glutamate decarboxylase 1 (Gad)-GAL4-2 and Gad-GAL4-5], Cha-GAL80 and Gad-GAL80 were raised on glucose-yeast-cornmeal medium at 24.5 ± 0.5 °C in a 12-h light:12-h dark (LD) cycle. All *pain* mutants, Cha-GAL4-19B, TH-GAL4, Ddc-GAL4 and UAS-*pain* were outcrossed for five or six generations to *white* flies with the Canton-S genetic background.

For Gad-GAL4 and Gad-GAL80 transformants, the 4.5 kb *Bam*HI/*Bam*HI Glutamic acid decarboxylase 1 (Gad1) 5'-flanking DNA was fused to GAL4 (Brand and Perrimon, 1993) and GAL80 (Lee & Luo 1999) cDNAs, respectively. The resultant fusion genes were inserted to the pCaSpeR2 vector. For Cha-GAL80, the lacZ portion of pCaSpeR-7.4 kb-lacZ (Kitamoto *et al.* 1992) was replaced with a GAL80 cDNA. The UAS-*pain* RNAi construct was created using a vector developed by Lee & Carthew (2003) with a transgene containing inverted repeats of partial *pain* cDNA (nucleotide positions 2280–2780) (Tracey *et al.* 2003). Transformed fly lines were established by injecting these DNA constructs in *white*

mutant embryos using standard procedure (Spradling & Rubin 1982). When the genetic background of the *white* mutants used for injection is not known, transformants were backcrossed to *white* flies with the Canton-S genetic background for at least 6 generations.

Virgin males or females were collected without anesthesia within 6 hours of eclosion and maintained in vials until experiments. All the experiments except for the temperature shift experiments were carried out during daytime between Zeitgeber time (ZT) 0 and ZT5 at 24.5 ± 0.5 °C in 50–60% relative humidity.

Observation of mating behavior

A pair of male and female flies was placed in an acrylic plastic observation chamber (15 mm diameter $\times$ 3 mm depth) using a manual aspirator. We observed mating behaviors of 10 pairs at the same time for 20 min, and at least 40 pairs were observed for each set of experiments. We measured the mating success rate, defined as the percentage of pairs that copulated during the 20 min period after placing male and female flies together in the observation chamber. Furthermore, we calculated the mean courtship latency and mean time to copulation (TC). The courtship latency is defined as the period between the moment the flies are placed in the chamber and the first courtship, and TC is defined as the time for male/female pairs to copulate after the male flies initiate courtship behavior. CS males were used in all the observations. All the flies used in the experiment were 4–6 days old.

Analysis of male courtship

A pair of male and female flies was placed in an observation chamber as described above. We then observed male courtship behavior for 10 min or until the moment of copulation and calculated the courtship index (CI). The CI is defined as the percentage of time spent courting in a given observation period. 15–20 pairs were observed for each group. CS females, *pain* mutant females, and CS males were used in the experiment. All the flies used in the experiment were 4–6 days old.

Quantification of locomotor activity by video-tracking

Intact single females were allowed to walk freely in an acrylic plastic observation chamber (15 mm diameter $\times$ 3 mm depth). 5-day-old virgin females were used. 4 individuals were simultaneously videotaped at a capture rate of 30 frames per second for 10 min. We used total distance moved (mm) as an index of locomotor activity. Traces were generated and total distance moved (mm) was calculated using Move-tr/2D 7.0 (Library Co., Tokyo, Japan).

Temperature shift experiments

UAS-*shi^{ts1}*/Gad-GAL4-2, UAS-*shi^{ts1}*/+ and +/Gad-GAL4-2 females were grown at 23.0 ± 0.5 °C (permissive temperature), and collected virgin females were also kept at the permissive temperature until 30–60 min before observation of mating behavior, at which point they were shifted to 30°C (restrictive temperature) until the end of the experiments (Fig. 9a). As a control, observation of mating behavior was carried out at 23.0 ± 0.5 °C. 5-day-old virgin females of these genetic constructs and 5-day-old CS males were used in the experiments.

Microscopy

GFP fluorescence was observed using a fluorescence microscope (Leica MZ 16F) and a confocal microscope (Nikon D-ECLIPSE C1 Si). For confocal microscopy, Z sections were collected at 1 μ m intervals and processed to construct projections through an extended depth of focus.

Statistical analysis

In most cases, the courtship latency, TC, and CI were not distributed normally. Thus, we carried out a log transformation of courtship latency and TC, and an arcsine transformation of CI. However, the transformed values did not show normal distribution.

Thus, we used non parametric tests (Kruskall-Wallis test and Mann-Whitney *U* test). Because total walking distances were distributed normally and homoscedasticity was evident, they were analyzed by the one-way ANOVA. We used computer software (SPSS 10.0J Base System for Windows) in these tests (SPSS Japan inc., Shibuyaku, Tokyo, Japan). The *G* test with Williams's correction was used for comparisons of mating success rates (Sokal & Rohlf 1995).

Results

pain mutant females copulate earlier than wild-type females

We observed mating behavior of wild-type Canton-S (CS) males and *painless* (*pain*) mutant females. We used mutant alleles, *pain*¹, *pain*³ and *pain*^{GAL4}. Both *pain*¹ and *pain*³ have a P-element insertion in the 5' untranslated exon of the *pain* gene. *pain*^{GAL4}, which contains a GAL4-enhancer trap insertion, was generated from *pain*¹ by P-element replacement (Al-Anzi *et al.* 2006; Tracey *et al.* 2003). The mating success rate, defined as the percentage of pairs that copulated was determined every 1 min (Fig. 1). The mating success rates of females homozygous for *pain*¹, *pain*³ and *pain*^{GAL4} were higher than those of CS females during the entire observation period (Fig. 1). An analysis of the 20 min data revealed significant differences between the accumulated mating success rates of *pain* mutant females and those of CS females (Fig. 1).

Next, we measured TC defined as the time for male/female pairs to copulate after males initiate courtship behavior. As shown in Fig. 2a, TC of *pain* mutant females was significantly shorter than that of CS females. In contrast to the shortened TC in *pain* mutant females, no significant difference was detected in the courtship latency (Fig. 2b) and CI (Fig. 2c) between CS and *pain* mutant females. These results show that *pain* mutant females do not elicit increased male courtship behavior than CS females and that the rapid copulation of virgin *pain* mutant females is due to their enhanced sexual receptivity.

In *D. melanogaster*, receptive females spent less time in locomotion before mating, and this female immobility is important for a successful copulation attempt (Markow & Hanson 1981). To examine whether the enhanced sexual receptivity in *pain* mutant females results from their reduced general locomotion, we measured the total walking distance within 10 min, as an indicator of locomotion, using the video-tracking system. No significant difference was found between CS and *pain* mutant females (Fig. 2d), suggesting that the enhanced sexual receptivity in *pain* mutant females does not simply result from general sluggishness.

pain expression in the pain mutant background restores the female sexual receptivity to the wild-type level

We used the GAL4/UAS system (Brand & Perrimon 1993) to examine if expression of wild-type *pain* rescues the enhanced sexual receptivity in *pain* mutant females. In transgenic flies with *pain*^{GAL4} on the second chromosome and UAS-*pain* on the third chromosome (*pain*^{GAL4}; UAS-*pain*), wild-type *pain* should be expressed in a *pain* mutant background with an expression pattern similar to that of *pain* in wild-type flies. UAS-*pain* females without *pain* mutations were used as a control. TC of females homozygous for *pain*^{GAL4}; UAS-*pain* was significantly longer than that of *pain*^{GAL4} mutant females, and no significant

difference was detected between females homozygous for UAS-*pain* and *pain^{GAL4}*; UAS-*pain* (Fig. 3a). This result confirmed that the enhanced sexual receptivity in *pain* mutant females is indeed caused by mutations in the *pain* gene. In addition, this result indicates that the GAL4-positive neurons in *pain^{GAL4}* are relevant to female sexual receptivity.

Induction of *pain* RNAi in *pain^{GAL4}*-positive neurons enhances female sexual receptivity

Next, we further explored whether *pain* expression plays a key role in female sexual receptivity using RNA interference (RNAi) to knock down the expression of *pain*. A UAS-*pain* RNAi line (2A) was used in combination with the GAL4/UAS binary expression system. In F1 females between *pain^{GAL4}* and UAS-*pain* RNAi (*pain^{GAL4}/UAS-pain* RNAi), *pain* expression should be repressed in GAL4-positive neurons in *pain^{GAL4}*. Females heterozygous for *pain^{GAL4}* (*pain^{GAL4}/+*) and UAS-*pain* RNAi (*+/UAS-pain* RNAi) were used as controls. TC of *pain^{GAL4}/UAS-pain* RNAi females was significantly shorter than that of control females, while no significant difference was detected between control females (*pain^{GAL4}/+* and *+/UAS-pain* RNAi) (Fig. 3b). This result indicates that the induction of *pain* RNAi in GAL4-positive neurons in *pain^{GAL4}* enhances female sexual receptivity.

Induction of *pain* RNAi in neuronal subsets enhances sexual receptivity

Several neural GAL4 drivers (Cha-GAL4-9A, Cha-GAL4-19B, TH-GAL4, Ddc-GAL4, Gad-GAL4-2 and Gad-GAL4-5) were used in combination with two UAS-*pain* RNAi lines (1A and 2A) to investigate the possible involvement of particular neurotransmitter systems in the sexual receptivity controlled by *pain*. Cha-GAL4, TH-GAL4, Ddc-GAL4 and Gad-GAL4 transgenes are composed of GAL4 cDNA fused to the regulatory region of the neurotransmitter-synthesizing enzymes for acetylcholine (Cha), dopamine (TH), dopamine and serotonin (Ddc) and GABA (Gad), respectively.

We found that the enhanced sexual receptivity in *pain* mutant females was phenocopied when *pain* RNAi was expressed in wild-type virgin females using two independent Cha-GAL4 lines, Cha-GAL4-9A and Cha-GAL4-19B (Fig. 4). TCs of 1A/Cha-GAL4-9A, 2A/Cha-GAL4-9A and 1A/Cha-GAL4-19B females were significantly shorter than that of *+/Cha-GAL4-9A* or *+/Cha-GAL4-19B* control females (Fig. 4a, b, gray boxes), while no significant difference was detected between GAL4 and UAS control females (Fig. 4a, b, white boxes). In contrast to Cha-GAL4, expression of *pain* RNAi using TH-GAL4 did not affect the female sexual receptivity (Fig. 5a). No significant difference in TC was detected between 1A/Ddc-GAL4 and *+/Ddc-GAL4* females (Fig. 5b), suggesting that the induction of *pain* RNAi driven by Ddc-GAL4 does not affect female sexual receptivity. However, TC of *+/Ddc-GAL4* females was significantly shorter than that of 1A/*+* females. Ddc-GAL4 line has been backcrossed to *white* line with Canton-S (CS) genetic background 6 generations. Thus, the increased female receptivity observed in the Ddc-GAL4 line is likely due to the P-element insertion itself. F1 females between Gad-GAL4 and UAS-*pain* RNAi showed enhanced sexual receptivity (Fig. 6a, b, gray boxes), while control females did not (Fig. 6a, b, white boxes). Thus, the induction of *pain* RNAi driven by Cha-GAL4 or Gad-GAL4 enhanced sexual receptivity.

pain^{GAL4} and Cha-GAL4 drive GFP reporter gene expression in the sensory neurons

Expression of wild-type *pain* in *pain^{GAL4}*-positive neurons rescued the enhanced sexual receptivity in *pain^{GAL4}* mutant females (Fig. 3a), and induction of *pain* RNAi in *pain^{GAL4}*-positive neurons enhanced female sexual receptivity (Fig. 3b), indicating that GAL4-positive neurons in *pain^{GAL4}* are responsible for the regulation of female sexual receptivity. Since olfactory (Gailey 1986; Kurtovic *et al.* 2007; Savarit *et al.* 1999; Tompkins *et al.* 1980) and auditory (Kyriacou & Hall 1982; Manning 1967; Tomaru *et al.* 2000; von Schilcher 1976) inputs are critically involved in female sexual receptivity, we examined whether *pain^{GAL4}*

drives *GFP* reporter gene expression in sensory neurons of the adult head. We generated transgenic flies (*pain^{GAL4} UAS-GFP*) with both *pain^{GAL4}* and *UAS-GFP* constructs in the second chromosome. In females homozygous for *pain^{GAL4} UAS-GFP*, *GFP* signal was detected in the sensory neurons of Johnston's organ (the fly's auditory organ) located within the second antennal segment, the maxillary palp (one of the *Drosophila* olfactory organs), taste neurons, and the reproductive tract (Fig. 7a-c and Fig. S1a). A similar expression pattern of *GFP* reporter gene was also observed in females heterozygous for *pain^{GAL4} UAS-GFP* (Fig. 7d). *GFP* signal was not detected in the olfactory organs within the third antennal segment in females homozygous and heterozygous for *pain^{GAL4} UAS-GFP* (Fig. 7a, d) in agreement with Al-Anzi *et al.* (2006).

In F1 females between *Cha-GAL4* and *UAS-GFP*, *GFP* reporter gene expression was detected in the second and third antennal segments, the maxillary palp, taste neurons (Fig. 8a, b) as reported by Salvaterra and Kitamoto (Salvaterra & Kitamoto 2001), and the reproductive tract (Fig. S1b). However, in *Gad-GAL4/UAS-GFP* females, *GFP* reporter gene expression was not detected in the second and third antennal segments, the maxillary palp, taste neurons in the proboscis (Fig. 8c, d), legs and wings (Data not shown), and the reproductive tract (Fig. S1c), suggesting that the enhanced sexual receptivity in F1 females between *UAS-pain* RNAi and *Gad-GAL4* does not result in the dysfunction of auditory and/or chemosensory reception.

To determine whether *GAL4*-expressing cells in the sensory neurons between *pain^{GAL4}* and *Cha-GAL4* overlap, we used a yeast transcriptional regulator, *GAL80*. *GAL80* blocks *GAL4* activity by binding to the C-terminal activation domain of *GAL4* (Ma & Ptashne, 1987). Thus, *Cha-GAL80* should suppress *GAL4* expression in cholinergic neurons. In *Cha-GAL80/pain^{GAL4} UAS-GFP* females, *GFP* reporter gene expression in the second antennal segment (Fig. 7e) and maxillary palp (Fig. 7f) was notably suppressed, suggesting that *GAL4*-expressing cells in these two sensory organs between *pain^{GAL4}* and *Cha-GAL4* overlap. On the other hand, *Gad-GAL80* in females heterozygous for *pain^{GAL4} UAS-GFP* did not affect *GFP* reporter gene expression (Figs 7g-i), suggesting that *GFP*-positive sensory neurons in *pain^{GAL4} UAS-GFP* females are not GABAergic.

pain mutant females seldom copulate with wild-type males without wings

To examine whether an auditory system is required for enhanced sexual receptivity in *pain* mutant females, we cut off wings of CS males and measured mating success rates (Fig. S2). CS females or *pain¹* mutant females were paired with CS males with or without wings (wingless or winged males, respectively) for 20 min. *pain¹* or CS females seldom copulated with wingless males (Fig. S2), suggesting that *pain* mutant females can detect these courtship songs produced by courting males and detection of courtship song triggers high mating success rate in *pain* mutant females.

Conditional disruption of neurotransmission in *Gad*-expressing neurons enhances female sexual receptivity

Because TRP channels are calcium-permeable cation channels that can modulate neuronal activities and affect neurotransmitter release, the enhancement of female sexual receptivity by *Gad-GAL4*-induced *pain* RNAi expression is possibly due to reduced synaptic transmission from *Gad*-expressing neurons. The temperature-sensitive Dynamin mutation, *shibire^{ts1}* (*shi^{ts1}*), can be used in combination with the *GAL4/UAS* binary expression system to deplete neurotransmitters in a cell-type-specific and temperature-dependent manner (Kitamoto 2001, 2002). To determine whether the suppression of neurotransmission in *Gad*-expressing neurons also affects the female sexual receptivity, *shi^{ts1}* was expressed by *Gad-GAL4* and the effects of conditional disruption of Dynamin function on female sexual

receptivity were examined by comparing TC at 23 and 30°C. In UAS-*shit^{ts1}*/Gad-GAL4-2 females, TC at 30°C was significantly shorter than that at 23°C, while no significant difference was detected in +/Gad-GAL4-2 or UAS-*shit^{ts1}*/+ (Fig. 9b). These data further demonstrate that GAL4-positive neurons in Gad-GAL4 play a key role in female sexual receptivity.

Although it is possible that the reduced TC in UAS-*pain* RNAi/Cha-GAL4 females is also caused by the suppression of neurotransmission in *pain*-expressing cholinergic neurons, we could not use UAS-*shit^{ts1}*/Cha-GAL4 females to test this possibility because they are paralyzed at 30°C as reported by Kitamoto (2001).

Discussion

pain mutations enhance sexual receptivity in *Drosophila* virgin females

In this study, we found a new role for the *Drosophila* Pain TRP channel in the regulation of female sexual receptivity. *pain* mutant females showed a high mating success rate and enhanced sexual receptivity (Fig. 1, 2a). There are at least two possible factors that contribute to induction of the shortened TC: (i) *pain* mutant females elicit more vigorous courtship behaviors in males than CS females; (ii) *pain* mutant females accept males more quickly. Because there was no significant difference in the courtship latency and CI between CS females and *pain* mutant females when CS males were used as sexual partner (Fig. 2b, c), we concluded that *pain* mutant females did not elicit more vigorous courtship behaviors in males than CS females. Hence, males are not the major factor for the enhanced sexual receptivity observed in the sexual behaviors of CS males and *pain* mutant females. Rather, the observed short TC is mainly attributable to *pain* mutant females. Because the expression of the wild-type *pain* gene in a *pain* mutant background restores female sexual receptivity to the wild-type level (Fig. 3a), the phenotype of the enhanced sexual receptivity is indeed caused by the *pain* mutations. Furthermore, we confirmed that knockdown of *pain* by RNAi in *pain^{GAL4}*-positive neurons also enhances female sexual receptivity (Fig. 3b), indicating that the reduced level of wild-type *pain* expression in *pain^{GAL4}*-positive neurons induces the hyper-receptive phenotype. In contrast to females, we confirmed that *pain* mutant males are fertile and their courtship does not show any major defects.

In vivo analysis of the Pain TRP channel revealed that Pain is required for thermal and mechanical nociception (Tracy *et al.* 2003) as well as avoidance of wasabi (Al-Anzi *et al.* 2006). Recently, Sokabe *et al.* (2008) found that Pain is directly activated by heat stimulation *in vitro*. Unexpectedly, however, Pain is completely insensitive to direct touch or the main ingredient of wasabi, allyl isothiocyanate (AITC), *in vitro* (Sokabe *et al.* 2008). Endogenous ligands of Pain relevant to the regulation of female sexual receptivity remain elusive. It will be very interesting to investigate the physiological mechanisms by which the activity of the Pain TRP channel in the nervous system of females is regulated in response to their interaction with courting males.

Several mutations induce a low sexual receptivity in virgin females (Hall 1994; Kerr *et al.* 1997; Ringo *et al.* 1991; Suzuki *et al.* 1997; Tompkins *et al.* 1982), while little is known about the mutations that lead to the enhanced sexual receptivity. Sakai & Kidokoro (2002) have reported that the induction of a repressor isoform of dCREB2, dCREB2-b, enhances female sexual receptivity as observed in *pain* mutants. dCREB2-b functions as a repressor of *Drosophila* CREB (Yin *et al.* 1995). Because the Pain TRP channel is permeable to Ca²⁺ (Sokabe *et al.*, 2008) and CREB can function as a Ca²⁺-inducible transcription factor (Lonze & Ginty 2002), it is possible that dCREB2-mediated gene expression is under the control of the Pain TRP channels and plays a role in maintaining the wild-type level of female sexual receptivity.

Female sexual receptivity is regulated by the Pain TRP channel expressed in neurons with particular neurotransmitter phenotypes

Induction of *pain* RNAi driven by Cha-GAL4 and Gad-GAL4 enhanced female sexual receptivity (Fig. 5, 7), suggesting that down-regulation of *pain* expression in cholinergic and/or GABAergic neurons leads to the enhanced sexual receptivity observed in *pain* mutant females. On the other hand, targeted expression of *pain* RNAi in dopaminergic and/or serotonergic neurons had no effect on female sexual receptivity (Fig. 6). In *Drosophila*, dopamine-depleted females are significantly less receptive to males than control females (Neckameyer 1998a), indicating the positive regulatory role of dopaminergic neurons in the regulation of female sexual receptivity. However, our results suggest that the dopaminergic regulation of female sexual receptivity is not mediated by the Pain TRP channel.

Results of two independent experiments using UAS-*pain* RNAi and UAS-*shits1* in combination with Gad-GAL4 suggest that the down-regulation of *pain* in Gad-GAL4-positive neurons leads to the suppression of GABAergic synaptic transmission, resulting in enhanced female receptivity. Sokabe *et al.* (2008) have recently reported that Pain is a cation channel with extremely high Ca^{2+} permeability. Hence, it is possible that *pain* mutations or knockdown of *pain* expression causes a decrease in the level of intracellular Ca^{2+} , which consequently reduces synaptic transmission in GAL4-expressing neurons in the Gad-GAL4 line and results in the enhanced sexual receptivity in females.

Possible anatomical sites in the sensory neurons and CNS responsible for Pain-mediated regulation of female sexual receptivity

We demonstrated that induction of *pain* RNAi in Cha-GAL4-positive neurons enhances female sexual receptivity (Fig. 4), suggesting that a decrease in the level of intracellular Ca^{2+} in *pain*-expressing cholinergic neurons also enhances female sexual receptivity. However, owing to the widespread gene expression in the nervous system directed by Cha-GAL4 (Salvaterra & Kitamoto 2001), the neural circuits or the sites in Cha-expressing cells relevant to *pain*-mediated regulation of female sexual receptivity are not yet determined. Possible anatomical sites that are important for *pain*-mediated regulation of female receptivity include sensory neurons involved in detecting signals that are transmitted from male flies during courtship. Because the courtship song generated by courting males stimulates female sexual receptivity (Kyriacou & Hall 1982; Manning 1967; Tomaru *et al.* 2000; von Schilcher 1976), the auditory neurons in the antennal second segment are the most likely candidate. Ai-Anzi *et al.* (2006) have observed the *pain*-reporter gene expression in Johnston's organs in the second antennal segment. We also confirmed that both *pain*^{GAL4} and Cha-GAL4 drive the GFP expression in those sensory neurons (Fig. 7a, d, 8a, b). Furthermore, Cha-GAL80 suppresses GFP expression in Johnston's organs in *pain*^{GAL4} females (Fig. 7e). These data suggest the expression of Pain in cholinergic auditory neurons. *pain*¹ or CS females seldom copulated with wingless males (Fig. S2). Thus, it is possible that the enhanced female sexual receptivity is caused by altered neural properties of the cholinergic auditory system in *pain* mutants. Alternatively, Cha-GAL4-positive neurons in the maxillary palp and/or adult CNS may also be responsible for the *pain*-mediated regulation of female sexual receptivity.

The *icebox* (*ibx*) gene encodes the L1-type cell adhesion molecule, Neuroglian. *ibx* mutant females show a low sexual receptivity and have defects in central brain structures including the mushroom bodies (MBs) that are composed of the higher order neurons of olfaction (Carhan *et al.* 2005; Kerr *et al.* 1997). Carhan *et al.* (2005) have reported that the expression of the wild-type *ibx* gene in the *ibx* mutant background partially rescues the female sexual receptivity without rescuing the brain structure phenotype (Carhan *et al.* 2005).

Furthermore, Neckameyer (1998b) has reported that female sexual receptivity is unaffected by the ablation of MBs. Thus, MBs may not be the important sites for female sexual receptivity, although it has been suggested that Pain TRP channels are expressed in MBs (Al-Anzi *et al.* 2006; Xu *et al.* 2006).

GFP reporter gene expression was not detected in the sensory neurons of Gad-GAL4/UAS-*GFP* female head (Fig. 8c, d) and Gad-GAL80 did not suppress the reporter gene expression in the sensory neurons of *pain^{GAL4}* females (Fig 7g-i). In addition, we confirmed that Gad-GAL4 drives GFP expression in the adult brain (Fig. S3) as reported by Mehren & Griffith (2006). Taken together, our results indicate that Gad-GAL4 drives gene expression mainly in the CNS. Thus, it is possible that GABAergic *pain^{GAL4}* neurons in the adult brain play a key role in the regulation of female sexual receptivity. Although the neural circuits or the regions of the brain relevant to *pain*-mediated regulation of sexual receptivity are still unknown in *Drosophila* virgin females, our results suggest that the Pain TRP channels not only in sensory neurons but also in the brain are involved in the manifestation of the wild-type level of female sexual receptivity in *Drosophila*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Cumulative mating success rate (%) in *pain* mutant and wild-type females *pain*¹ (crosses), *pain*³ (squares), *pain*^{GAL4} (triangles), and CS (circles) females and CS males were used. Observation period was 20 min. 40 pairs were observed for each genotype. *, $P < 0.05$; **, $P < 0.01$.

Figure 2. Time to copulation, courtship latency, courtship index, and locomotion in *pain* mutant and wild-type females

(a) Time to copulation (sec), (b) male courtship latency (sec), (c) male courtship index (%), (d) total walking distance (mm). In all experiments, *pain*¹, *pain*³, *pain*^{GAL4}, and CS females and CS males were used. In each box plot, the box encompasses the interquartile range, a line is drawn at median, and error bars correspond to the 10th and 90th percentiles. Each square within a box is the mean. *N*, sample size; ***, $P < 0.001$.

Figure 3. *pain* expression in *pain^{GAL4}*-positive neurons is involved in the regulation of female sexual receptivity

(a) Time to copulation (sec) in *pain^{GAL4}*, *UAS-pain* and *pain^{GAL4}; UAS-pain* females. CS males were used. The observation period was 20 min. *N*, sample size; *, $P < 0.05$; **, $P < 0.01$; NS, not significant. (b) Time to copulation (sec) in *pain^{GAL4}/+*, *UAS-pain RNAi* (2A)/+ and *pain^{GAL4}/UAS-pain RNAi* (2A) females. CS males were used. The observation period was 20 min. *N*, sample size; *, $P < 0.05$; **, $P < 0.01$; NS, not significant.

Figure 4. Induction of *pain* RNAi driven by Cha-GAL4 enhances sexual receptivity

Two UAS-*pain* RNAi lines (1A and 2A), Cha-GAL4-9A (a) and Cha-GAL4-19B (b) were used. The observation period was 20 min. White boxes indicate control females, and gray boxes indicate F1 females between Cha-GAL4 and UAS-*pain* RNAi. *N*, sample size; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

Figure 5. Induction of *pain* RNAi driven by TH-GAL4 or Ddc-GAL4 does not affect female sexual receptivity

Two UAS-*pain* RNAi lines (1A and 2A), TH-GAL4 (a) and Ddc-GAL4 (b) were used. The observation period was 20 min. White boxes indicate control females, and gray boxes indicate F1 females between GAL4 lines and UAS-*pain* RNAi. *N*, sample size; **, $P < 0.01$; NS, not significant.

Figure 6. Induction of *pain* RNAi driven by Gad-GAL4 enhances sexual receptivity

Two UAS-*pain* RNAi lines, 1A and 2A, Gad-GAL4-2 (a) and Gad-GAL4-5 (b) were used. The observation period was 20 min. White boxes indicate control females, and gray boxes indicate F1 females between Gad-GAL4 and UAS-*pain* RNAi. *N*, sample size; *, $P < 0.05$; ***, $P < 0.001$; NS, not significant.

Figure 7. *pain^{GAL4}* drives GFP reporter expression in the sensory organs

We observed GFP reporter expression using females homozygous for *pain^{GAL4} UAS-GFP* (a-c), females heterozygous for *pain^{GAL4} UAS-GFP* (d), *Cha-GAL80/pain^{GAL4} UAS-GFP* females (e, f) and *Gad-GAL80/pain^{GAL4} UAS-GFP* females (g-i). (a-i) are confocal microscope images. (a) and (d) show adult head. (b) shows the second segment of antenna. (c) and (h) show the maxillary palp. (e) and (g) show the second and third antennal segments. (f) shows the maxillary palp and taste neurons. (i) shows taste neurons. Arrowheads show the second antennal segment. Asterisks show the third antennal segment. Arrows show the maxillary palp. Triangles show taste neurons.

Figure 8. GFP reporter expression in Cha-GAL4 and Gad-GAL4 lines

We observed GFP reporter expression using F1 females between GAL4 lines and UAS-*GFP*. All pictures are confocal microscope images. (a) Cha-GAL4-9A/UAS-*GFP* females. (b) Cha-GAL4-19B/UAS-*GFP* females. (c) Gad-GAL4-2/UAS-*GFP* females. (d) Gad-GAL4-5/UAS-*GFP* females. Arrowheads show the second antennal segment. Asterisks show the third antennal segment. Arrows show the maxillary palp. Triangles show taste neurons.

Figure 9. Disruption of Dynamin function in Gad-GAL4-positive neurons enhances sexual receptivity

(a) Experimental paradigms of temperature shift are indicated. (b) UAS-*sh^{ts1}*/+, +/Gad-GAL4-2 and UAS-*sh^{ts1}*/Gad-GAL4-2 females and CS males were used. *N*, sample size; *, $P < 0.05$; NS, not significant.